MOLECULAR PHARMACOLOGY, 16, 719–728

Quantitation and Characterization of Human Platelet *Alpha*-Adrenergic Receptors Using [^{3}H]Phentolamine

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(Received November 9, 1978)
(Accepted June 7, 1979)

SUMMARY

STEER, M. L., J. KHORANA AND B. GALGOCI. Quantitation and characterization of human platelet *alpha*-adrenergic receptors using [^{3}H]phentolamine. *Mol. Pharmacol.* **16**: 719–728, (1979).

The binding of [^{3}H]phentolamine to human platelet membranes was studied, and this ligand was found to bind to a single class of noncooperative sites with a dissociation constant (K_d) of 12 ± 3 nM. At saturation, 0.165 ± 0.060 pmoles were bound per mg protein, and each platelet was noted to possess 260 ± 95 binding sites. [^{3}H]phentolamine binding was rapid ($t_{1/2}$ < 15 sec) and reversible ($t_{1/2}$ for dissociation <30 sec) at 30°. [^{3}H]phentolamine binding was stereospecifically inhibited by epinephrine. Inhibition of [^{3}H]phentolamine binding by catecholamines occurred with an order of potency of epinephrine > norepinephrine > isoproterenol and propranolol (1 μM) did not inhibit [^{3}H]phentolamine binding. These observations indicate that [^{3}H]phentolamine binds to sites having the characteristics of α-adrenergic receptors. Dihydroergocryptine was also noted to cause time and concentration-dependent inhibition of [^{3}H]phentolamine binding. Comparison of these data to those previously reported using [^{3}H]dihydroergocryptine as the binding probe (Newman, K. D., Williams, L. T., Bishopric, N. H. *et al.* J. Clin. Invest. **61**: 395–402, 1978; Alexander, R. W., Cooper, B. and Handin, R. I. J. Clin. Invest. *61*, 1136–1144, 1978) suggests that both [^{3}H]phentolamine and [^{3}H]dihydroergocryptine interact with the same binding sites. Dopamine and serotonin were found to inhibit [^{3}H]phentolamine binding (K_d 1.9 and 15.1 mM respectively) indicating either that phentolamine can bind to receptors for dopamine and serotonin or these ligands bind to the platelet α-receptor. The binding of [^{3}H]phentolamine to platelet membranes was not altered when GTP (0.01 mM) was present, but the nucleotide caused a tenfold shift in the inhibition curve for epinephrine, indicating that epinephrine was less avidly bound in the presence of GTP.

INTRODUCTION

Catecholamines interact with a wide variety of cells and evoke a number of physiological responses, which have been classified by Ahlquist (1) as either α or β depending on (a) the order of potency with which the response is elicited by the catecholamines, and (b) the agents that are capable of blocking the response. Thus, catecholamines elicit α-responses with a potency order of epinephrine > norepinephrine > isoproterenol, and α-responses are blocked by phentolamine, phenoxybenzamine, and the ergot alkaloids. *Beta*-responses are in-

This work was supported by grant #HL20956 from the National Heart, Lung, and Blood Institute.

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[2] Recipient of Research Career Development Award #NS00160 from the National Institute of Neurological and Communicative Disorders and Stroke.

0026-895X/79/060719-10$02.00/0

duced by catecholamines with an order of potency of isoproterenol > epinephrine > norepinephrine and are blocked by agents such as propranolol. The catecholamines are believed to interact with their target cells by binding to surface sites called receptors. The development of radioactively labeled β-adrenergic antagonists such as [^{3}H]propranolol (2), [^{3}H]alprenolol (3) and [^{125}I]hydroxybenzylpindolol (4) have facilitated in-depth studies of the β-adrenergic receptor and characterization of the structural requirements for binding to that site. For the most part, β-adrenergic agonists and antagonists are structurally similar, suggesting rather strict specificity for binding to the β-receptor. In contrast, α-adrenergic agonists and antagonists appear to have great structural differences (5).

Human platelets aggregate in response to catecholamine stimulation, and this aggregation can be inhibited by phentolamine, suggesting that catecholamine-induced aggregation is an α-adrenergic event (6–8). Two groups of investigators have recently reported studies using [^{3}H]dihydroergocryptine which show that this agent binds to human platelet sites having the characteristics of α-adrenergic receptors (9, 10). In this communication, we report similar studies using a tritiated form of the classical α-adrenergic antagonist phentolamine. To our knowledge, this is the first report of α-receptor binding studies using [^{3}H]phentolamine as the radiolabeled probe.

MATERIALS

l-epinephrine hydrochloride, *l*-norepinephrine bitartrate, *l*-phenylephrine bitartrate, yohimbine, dopamine, serotonin, *l*-isoproterenol bitartrate, and lactoperoxidase were obtained from Sigma Chemical Co., St. Louis, Mo. Phentolamine hydrochloride was from Ciba-Geigy, and dihydroergocryptine from Sandoz. *l*- and *d*-epinephrine bitartrate used for stereospecificity experiments were gifts from Sterling-Winthrop Laboratories. Propranolol was from Ayerst, azapetine from Hoffman-LaRoche, clonidine from Boehringer Ingelheim, and methoxamine from Burroughs-Wellcome. Spiperone and pimozide were from Janssen Pharmaceuticals, haloperidol and dibozane from McNeil Laboratories, and chlorpromazine and phenoxybenzamine from Smith Kline and French. All other reagents were of the greatest purity commercially available. Protosol, Econofluor, and Na[^{125}I] were from New England Nuclear. [^{3}H]phentolamine (23.0 Ci/mM) was synthesized and kindly donated by Dr. N. Wigger, Radiosynthesis Laboratory, Ciba-Geigy Pharmaceuticals, Basal, Switzerland.

METHODS

Platelet Preparation. All experiments were performed using platelets obtained from donors who had ingested no drugs for at least the preceding 2 weeks. Blood was collected by venipuncture through siliconized needles and polyvinyl chloride tubing into polypropylene tubes containing 3/20 vol of acid citrate anticoagulant (11). Platelet rich plasma (PRP)[3] was collected after centrifugation (200 × *g*, 20 min) at room temperature, and platelet membranes were prepared from fresh PRP using a modification of the glycerol-lysis technique of Barber and Jamieson (12). Platelets were pelleted from PRP by centrifugation (4,300 × *g*, 10 min, 4°) and resuspended in cold (4°) pH 7.6 buffer containing 0.01 M Tris-HCl, 0.001 M EDTA and 0.15 M NaCl. The platelets were again pelleted by centrifugation (4,300 × *g*, 10 min, 4°), resuspended in 1 ml of the above buffer, and layered over a linear glycerol gradient (0–40% in 0.15 M NaCl, 4°). The pletelets were then slowly pelleted through the glycerol gradient by centrifugation (2000 × *g*, 30 min, 4°) in a swinging bucket rotor, resuspended and lysed in 10 ml of pH 7.6 buffer containing 0.01 M Tris-HCl, 0.001 M EGTA, 0.25 M sucrose and 0.001 M DTT. The platelet membranes were pelleted by centrifugation (5,860 × *g*, 10 min, 4°), and this pellet was resuspended in pH 7.6 buffer containing 0.05 M Tris-HCl, 0.005 M $MgCl_2$, 0.001 M EDTA, and 0.001 M DTT and used immediately.

[^{3}H]Phentolamine Binding. Prelimi-

[3] Abbreviations used are: PRP, platelet rich plasma; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin.

nary experiments revealed that the [^{3}H]-phentolamine co-migrated with nonradioactive phentolamine and was greater than 90% pure when tested by thin layer chromatography in three separate systems (methylene chloride-NH_3 (saturated)/methanol (5:1); benzene/dioxane/ethanol/diethylamine (50:30:10:5); and methylene chloride/2-propylamine/ethanol (20:4:1)) using silica gel plates. Binding studies were conducted using freshly prepared platelet membranes in a final volume of 0.1 ml. Unless otherwise specified, incubations were for 5 min at 30°. Final concentrations of agents in the assay mixture were: Tris-HCl (pH 7.6), 50 mM; $MgCl_2$, 5 mM; EGTA, 1 mM; DTT, 0.1 mM; BSA, 0.025%; and platelet protein, 1–2 mg/ml. The incubation was terminated by addition of 3.5 ml of cold (4°) pH 7.6 buffer containing 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EGTA and 0.1 mM DTT. Samples were rapidly (<15 sec) filtered through Whatman GF/A filters washed 3 times with 3.5 ml of the above buffer. The filters were dissolved by incubation (30 min, 60°) in 1.3 ml Protosol, neutralized by addition of 50 μl glacial acetic acid, and counted in a Packard liquid scintillation counter after addition of 10 ml Econofluor. Nonspecific binding was determined using samples incubated in the presence of 1 mM nonradioactive phentolamine or 0.01 mM *l*-epinephrine, and this value (50–70% of total binding with 20 nM [^{3}H]-phentolamine) was subtracted from that obtained in the absence of excess cold phentolamine or epinephrine to determine the amount of specific binding. The identity of specifically bound [^{3}H]phentolamine was determined by eluting dried filters with toluene-ethanol (1:1), evaporating the solvent and performing thin layer chromatography as described above. Under these conditions, the eluted [^{3}H]compound co-migrated with [^{3}H]phentolamine.

Protein determinations. Platelet membrane protein was assayed using the method Lowry *et al.* (13) and BSA as the standard. An aliquot of washed platelets was counted by phase contrast microscopy, radio-iodinated using ^{125}I and lactoperoxidase according to the method of Phillips (14), counted in a Nuclear Chicago gamma counter, and processed through the glycerol gradient and washing steps. Using the known cpm/platelet and cpm/mg protein, it was determined that 3.8×10^8 platelets yield 1 mg of protein. Non-iodinated samples were prepared in parallel to the above to determine the number of binding sites per platelet.

RESULTS

Binding of [^{3}H]phentolamine to platelet membranes. The binding of [^{3}H]phentolamine to platelet membrane preparations is shown in Figure 1. Maximal specific binding was found to be 0.165 ± 0.060 pmoles/mg protein (mean ± SD of 7 separate experiments), and the K_d for [^{3}H]phentolamine was noted to be 12 ± 3 nM. Scatchard plots (15) of the binding data consistently gave a straight line indicating that only one class of binding sites were present (Figure 1B). A Hill plot (16) of the data had a slope of 1.0, indicating that [^{3}H]phentolamine was noncooperatively bound. Since 1 mg of protein was obtained from 3.8×10^8 platelets and 1 mg of protein was found to bind 0.165 ± 0.060 pmoles of [^{3}H]phentolamine, we have calculated that there are 260 ± 95 [^{3}H]phentolamine binding sites per platelet. Binding of [^{3}H]phentolamine was linearly related to the protein concentration up to a protein concentration of 1–2 mg/ml (not shown).

Time course of [^{3}H]phentolamine binding. [^{3}H]phentolamine was bound with a half-time of less than 30 seconds (Fig. 2A). Binding was reversible with dissociation noted to have a half-time of less than 30 seconds (Fig. 2B). The time dependence for dissociation after dilution in the presence or absence of 0.01 mM epinephrine was similar (not shown), supporting the conclusion (see above) that binding was noncooperative (17). The half-times for binding and dissociation at 4° were also short (30–45 sec, not shown). Because of the rapidity of binding and dissociation, these kinetic constants could not be accurately determined.

Inhibition of [^{3}H]phentolamine binding by competing ligands. The binding of [^{3}H]phentolamine was stereospecifically inhibited by epinephrine (Fig. 3), and the *l*-stereoisomer was found to be 13–17 (mean

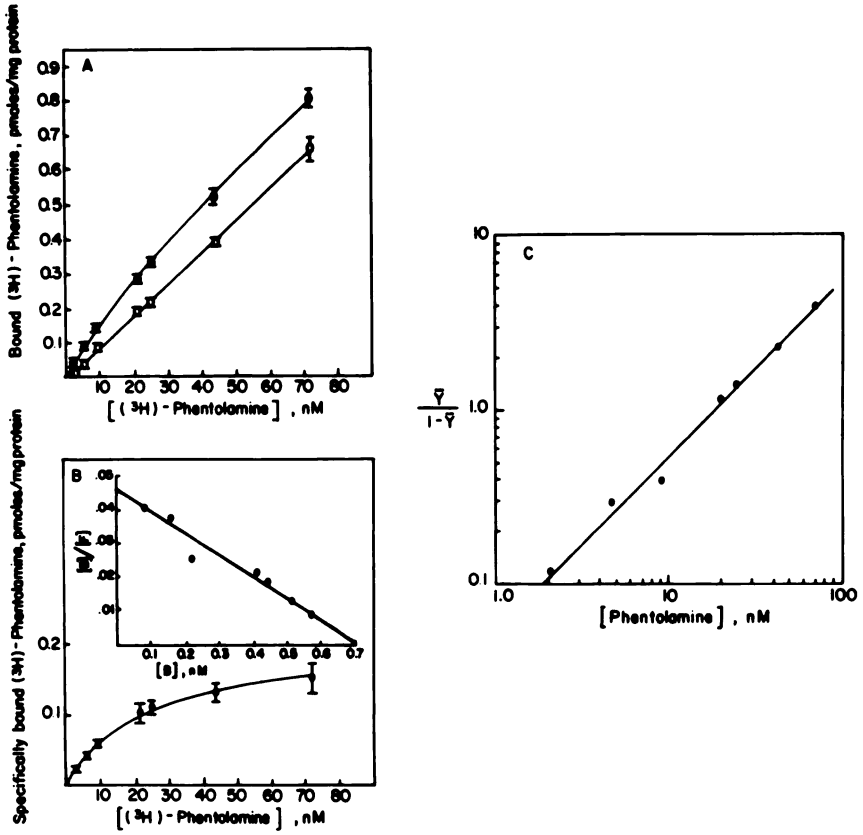

FIG. 1. *Binding of [³H]phentolamine*

(A) Platelet membranes (0.8 mg/ml) were incubated for 5 minutes at 30° with increasing concentrations of [³H]phentolamine in the presence (○——○) or absence (●——●) of 1 μM nonradioactive phentolamine. (B) *Specifically bound ³H-phentolamine.* Data from panel (A) are re-plotted after subtraction of values obtained in the presence of 1 μM nonradioactive phentolamine (nonspecific binding). Inset is Scatchard plot (15) of data where [B] and [F] represent the concentration of specifically bound and free [³H]phentolamine respectively. The slope of this line was used to calculate K_d for phentolamine, which was found to be 16 nM (r = 0.98). Maximal binding is 0.185 pmoles/mg protein. (C) *Hill plot of binding data.* Data from panel B are replotted according to the method of Hill (16). The slope (n_H) was found to be 1.0 (r = 0.99). Data are representative of 7 such experiments. Values represent mean, and vertical bars the standard deviation of quadruplicate determinations.

15, 6 experiments) times as potent as the *d*-stereoisomer. Concentration-dependent inhibition of [³H]phentolamine binding by various α-adrenergic agonists and antagonists is shown in Figures 4–6. The K_d values for ligands inhibiting [³H]phentolamine binding were calculated according to the method of Cheng and Prusoff (18) and are shown in Table 1. Adrenergic agonists inhibited [³H]phentolamine binding with an order of potency of clonidine > epinephrine > norepinephrine > phenylephrine > isoproterenol = methoxamine. The order of potency for α-antagonists was yohimbine > phentolamine ≥ dibozane ≥ dihydroergocryptine > phenoxybenzamine > azapetine. Propanolol at concentrations as high as 1 μM failed to inhibit [³H]phentolamine binding. The inhibition of [³H]phentolamine binding by dihydroergocryptine was found to be time and concentration dependent (Fig. 6).

Dopamine (K_d = 1.9 μM) and serotonin (K_d = 15.1 μM) inhibited [³H]phentolamine binding (Table 1), but addition of either agent in the presence of a maximally in-

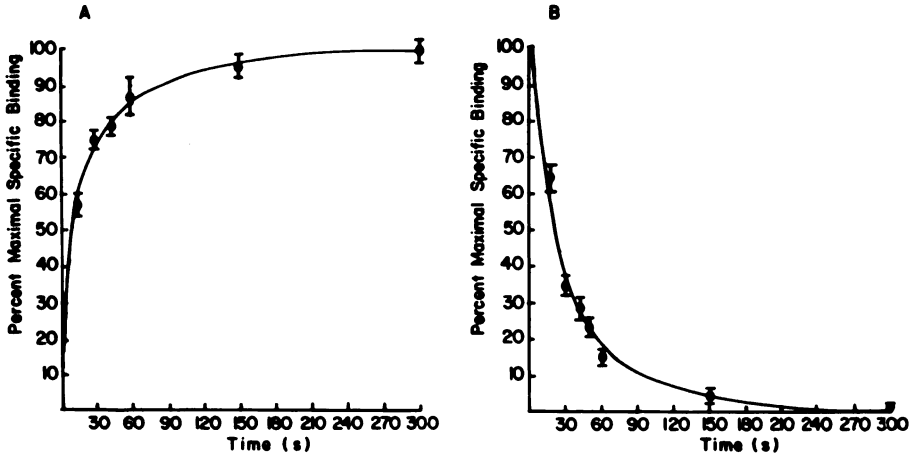

FIG. 2. *Time dependence of [^{3}H]phentolamine binding and dissociation*

(A) Platelet membranes were incubated for varying times at 30° with 40 nM [^{3}H]phentolamine in the presence or absence of 1 μM nonradioactive phentolamine. Data represent mean values ± SD after subtraction of nonspecific from total binding. Maximal binding (100%) in this experiment was 0.12 pmoles/mg protein. (B) Platelet membranes were incubated with [^{3}H]phentolamine (40 nM) for 5 min at 30° and then made 1 μM in nonradioactive phentolamine. After varying periods of additional incubation at 30°, samples were filtered and counted as described in the text. Values represent mean, and vertical bars represent the standard deviation of triplicate determinations. The data is representative of 3 such experiments.

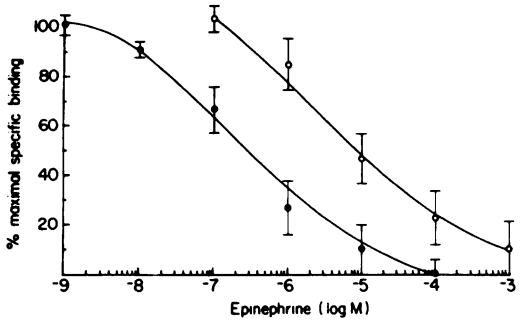

FIG. 3. *Stereospecificity of the inhibition of [^{3}H]phentolamine binding by epinephrine*

Platelet membranes were incubated with 20 nM [^{3}H]phentolamine and varying concentrations of either *l*-epinephrine (●——●) or *d*-epinephrine (○——○) for 5 min at 30°. Additional incubations were performed with [^{3}H]phentolamine with or without 1×10^6 nonradioactive phentolamine to determine specific binding. Data represent mean, and vertical bars represent the standard deviation of triplicate determinations and are representative of 6 separate experiments.

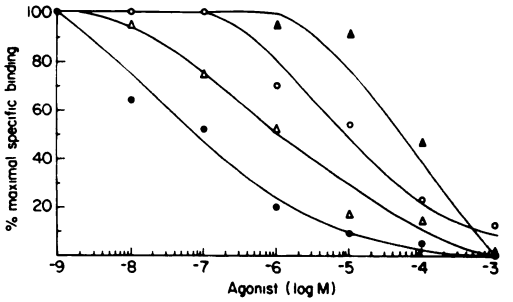

FIG. 4. *Inhibition of [^{3}H]phentolamine binding by adrenergic agonists*

Platelet membranes were incubated with 20 nM [^{3}H]phentolamine for 5 min at 30° in the presence of varying concentrations of: clonidine (●——●), *l*-norepinephrine (△——△), *l*-phenylephrine (○——○), or *l*-isoproterenol (▲——▲). Additional incubations were performed with 20 nM [^{3}H]phentolamine in the presence or absence of 0.01 mM *l*-epinephrine to determine specific binding. Data which are representative of 2 such experiments show mean values from triplicate determinations and the standard deviation never exceeded ±4%.

hibiting concentration of epinephrine (0.01 mM) did not cause inhibition of [^{3}H]phentolamine binding in excess of that observed with the same concentration of epinephrine alone (not shown). Inhibition of [^{3}H]phentolamine binding was also noted following addition of neuroleptics including spiperone (K_d = 0.6 μM), haloperidol (K_d = 5.4 μM), chlorpromazine (K_d = 10.3 μM) and pimozide (K_d = 39.8 μM) (Table 1).

The data from experiments such as those shown in Figs. 4, 5 and 6 were used to

calculate the Hill coefficients (16) for the inhibition of [^{3}H]phentolamine binding by agonists and antagonists. These values are shown in Table 1. For the most part, Hill coefficients less than 1.0 were noted although values of 0.9–1.2 were obtained for isoproterenol, yohimbine, phenoxybenzamine, serotonin, chlorpromazine and haloperidol.

Effects of GTP and divalent cations. Binding of [^{3}H]phentolamine (i.e., K_d, maximal binding) was not altered by addition of 0.01 μM GTP (not shown). In contrast, the concentration-dependent inhibition of [^{3}H]phentolamine binding induced by epinephrine was shifted to the right so that higher concentrations of epinephrine were required to inhibit [^{3}H]phentolamine binding in the presence of GTP (Fig. 7). This resulted in a calculated 5–20-fold (mean = 10, 3 experiments) decrease in the affinity for epinephrine as a result of the addition of 0.01 mM GTP. This effect of GTP was maximal with 0.01 mM and half maximal with 7 μM GTP (not shown). The binding of [^{3}H]phentolamine and its inhibition by agonists and antagonists was not changed when the Mg^{2+} concentration in the assay was varied from 0.1–10 mM or Ca^{2+} (10 mM) was added, (not shown).

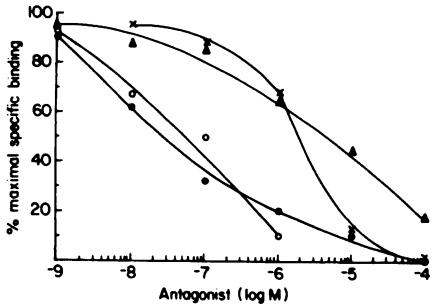

FIG. 5. *Inhibition of [^{3}H]phentolamine binding by adrenergic antagonists*

Details are the same as for Figure 4 legends. Symbols are: (●——●), phentolamine; (○——○), dibozane; (▲——▲), azapetine; and (×——×), phenoxybenzamine.

DISCUSSION

Phentolamine is a widely used adrenergic antagonist, and blockade of an adrenergic event by phentolamine has been considered one of the basic characteristics by which that event can be classified as an α-adrenergic phenomenon. In this communication, we have reported studies of [^{3}H]phen-

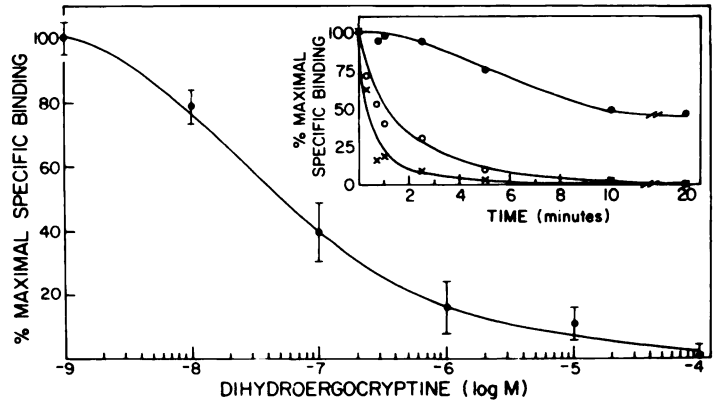

FIG. 6. *Inhibition of [^{3}H]phentolamine binding by dihydroergocryptine*

Platelet membranes were incubated at 30° in the dark with [^{3}H]phentolamine (20 nM) with or without epinephrine (0.01 μM) and varying concentrations of dihydroergocryptine. Results represent mean of 3 separate experiments, each performed with 3–4 replicate samples at each condition. Vertical bars represent ± SEM of the 3 separate experiments. *Inset: Time dependence of dihydroergocryptine inhibition ^{3}H-phentolamine binding.* Platelet membranes were pre-incubated for 5 min at 30° with [^{3}H]phentolamine (20 nM), with or without epinephrine (0.01 mM). Following preincubation, dihydroergocryptine at final concentrations of 0.03 μM (●——●), 0.3 μM (○——○), and 1 μM (×——×) was added and the samples incubated for varying periods of time at 30° in the dark. Results represent mean values of quadruplicate samples varying by ±10% and are representative of 2 such experiments.

TABLE 1

Binding of ligands to platelet membranes

Ligand	K_d (μM)[a] Mean SD	Hill coefficient[c] n_H
Agonists		
clonidine	0.020 ± 0.003	0.5
l-epinephrine	0.14 ± 0.02	0.6
d-epinephrine	2.18 ± 0.89	—
l-norepinephrine	0.44 ± 0.07	0.6
phenylephrine	2.8 ± 0.6	0.5
l-isoproterenol	26.4 ± 2.2	1.1
methoxamine	28.3 ± 4.0	—
Antagonists		
yohimbine	0.002 ± 0.001	1.0
phentolamine[b]	0.012 ± 0.003	1.0
dibozane	0.030 ± 0.010	0.6
dihydroergocryptine	0.059 ± 0.010	0.6
phenoxybenzamine	0.58 ± 0.12	1.0
azapetine	1.60 ± 0.40	0.4
propranolol	ND	—
Others		
spiperone	0.60 ± 0.20	—
dopamine	1.90 ± 0.70	0.7
haloperidol	5.37 ± 2.30	0.9
chlorpromazine	10.30 ± 2.50	0.9
serotonin	15.07 ± 1.81	1.2
pimozide	39.8 ± 10.5	—

[a] Data derived from studies of the inhibition of [^{3}H]phentolamine binding by ligand and calculated according to the method of Cheng and Prusoff (18), using the relationship $K_d = S_{0.5}/(1 + S/K_m)$ where $S_{0.5}$ = concentration of ligand causing 50% displacement of specific binding, S = concentration of [^{3}H]phentolamine (0.02 μM) and K_m = dissociation constant for [^{3}H]phentolamine (0.012 μM) obtained from direct [^{3}H]phentolamine binding studies. ND indicates no displacement at a concentration of 1μ M. Values represent mean ± SD for results from 2 or more experiments with each ligand in which triplicate determinations of displacement at each ligand concentration were obtained.

[b] Data derived from Scatchard plots (15) of binding using increasing concentrations of [^{3}H]phentolamine. Value represents mean ± SD of 7 separate experiments each performed in triplicate.

[c] Hill coefficients (16) were calculated from experiments such as those shown in Figs. 3–6.

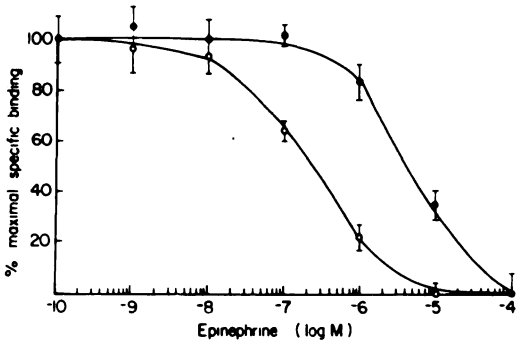

FIG. 7. *Effect of GTP on epinephrine inhibition of [^{3}H]phentolamine binding*

Platelet membranes were incubated with [^{3}H]phentolamine (20 nM) and varying concentrations of epinephrine in the presence (●——●) or absence (○——○) of GTP (0.1 mM) and specifically bound [^{3}H]phentolamine measured as described in the text. Results represent mean, and vertical bars, the SD of triplicate measurements in one experiment and are representative of the results obtained in 3 such experiments.

tolamine binding to human platelet membranes. [^{3}H]phentolamine was found to bind rapidly and reversibly. The binding of [^{3}H]phentolamine was inhibited by epinephrine (*l*-epinephrine/*d*-epinephrine potency ratio = 15) and other catecholamines (potency order: epinephrine > norepinephrine > isoproterenol) but not by propranolol (1 μM). These observations indicate that [^{3}H]phentolamine binds to human platelet membrane sites having the characteristic of α-adrenergic receptors. Using the level of maximal specific [^{3}H]phentolamine bound at saturating concentrations (0.165 ± 0.060 pmoles/mg), we have calculated that each platelet possesses 260 ± 95 α-adrenergic receptors. As binding and dissociation of [^{3}H]phentolamine were very rapid, accurate assessment of the kinetic constants for these processes was not possible. In addition, because of the rapid dissociation noted to occur even at 4°, it is likely that some of the specifically bound [^{3}H]phentolamine was lost during the washing phase of the binding assay and that the calculated number of receptors/platelet is higher than the actual value. However, values for the number of binding sites/platelet obtained by others (9, 10) using the slowly and only partially dissociable ligand [^{3}H]dihydroergocryptine (100–220 sites/platelet) are very close to that reported in this communication.

The binding of [^{3}H]phentolamine was inhibited by a number of α-adrenergic agonists and antagonists. Clonidine, a 2-substi-

tuted imidazoline, was found to be the most potent agonist. In contrast, methoxamine was 500 times less potent than epinephrine and was equal in potency to the classical β-agonist isoproterenol. Yohimbine, an indolalkylamine, was the most potent antagonist tested. The benzodioxan dibozane and the ergot alkaloid dihydroergocryptine were approximately equipotent to the 2-substituted imidazoline phentolamine and each was more potent than either phenoxybenzamine, a haloakylamine, or azapetine.

The binding of [^{3}H]phentolamine was inhibited by dopamine and serotonin. Since platelets are known to possess an uptake system for serotonin (19, 20), which can also facilitate dopamine uptake, it is possible that inhibition of [^{3}H]phentolamine binding by these agents indicates that they are inhibiting [^{3}H]phentolamine binding to sites that are part of the serotonin uptake system. It is also possible that platelets possess serotonin and dopamine receptors that are not related to either the α-adrenergic receptor or the serotonin uptake system but that can also bind phentolamine. Finally, serotonin and dopamine inhibition of [^{3}H]phentolamine binding may indicate that these agents bind to the platelet α-adrenergic receptor. This last explanation would seem the most plausible for the following reasons: (a) serotonin was less potent than dopamine in inhibiting [^{3}H]phentolamine binding, although the reverse would be expected if the serotonin uptake system were being labeled with [^{3}H]phentolamine (21); (b) inhibition of [^{3}H]phentolamine binding by maximal concentrations of epinephrine (0.1 mM) was not increased by the addition of high concentrations (1 mM) of either serotonin or dopamine, although enhanced inhibition might be expected if [^{3}H]phentolamine was bound to receptors for these ligands in addition to α-receptors; (c) spiperone and pimazide were found to be relatively weak inhibitors (calculated K_d = 0.6 and 39.8 μM respectively) of [^{3}H]phentolamine binding, although they have been reported to be potent inhibitors of dopamine and serotonin receptors in other tissues (22–24) and (d) both dopamine and serotonin are capable of promoting platelet aggregation (25, 26), which is what might be expected if they were to interact with the α-receptor. [^{3}H]-phentolamine was found to bind to a single class of sites and the Hill coefficient (16) for [^{3}H]phentolamine binding was 1.0, indicating that the binding is noncooperative. Some of the agents which inhibit [^{3}H]phentolamine binding also appeared to bind in a noncooperative fashion since Hill coefficients calculated from the inhibition of binding data approximated 1.0 (Table 1, isoproterenol, yohimbine, phenoxybenzamine, serotonin, chlorpromazine and haloperidol). On the other hand, the Hill coefficients for other agents were significantly less than 1.0. Although this might indicate that these ligands are bound in a negatively cooperative manner to the platelet α-receptor, it is more likely that the low Hill coefficients merely indicate that these ligands can occupy many types of membrane binding sites, only one of which is the α-receptor.

It is noteworthy that ligands of diverse chemical structures appear to interact with the platelet α-adrenergic receptor by inhibiting [^{3}H]phentolamine binding. In contrast, β-adrenergic agonists and antagonists are structurally quite similar and binding studies using radioactively labeled β-antagonists (2–4) have defined the structural requirements for binding to the β-receptors and have been used to "map" the β-receptor. The diversity of compounds which appear to bind to α-adrenergic receptors has complicated studies designed to elucidate α-receptor topography and even suggested that α-adrenergic agents do not interact at common binding sites (5). For example, recent studies using brain tissue have been interpreted as indicating that α-agonists and antagonist sites are not the same (27, 28), and the mixed agonist-antagonist dihydroergocryptine was found to bind to both agonist and antagonist sites. As noted by Triggle (5), the frequent observation that α-agents display competetive effects in either eliciting or blocking a physiological event does not establish, by itself, that these agents bind to a common site since similar responses might be observed if these agents were bound to geographically distinct but functionally interacting sites.

Even direct binding studies demonstrating competitive interactions among ligands could be observed under these conditions. On the other hand, there is no *a priori* reason why agents of dissimilar structure might not interact with a single binding site if one assumes that these agents bind to differing but overlapping areas within one binding domain. It is of interest, therefore, that the presently reported studies using the pure α-antagonist [^{3}H]phentolamine have yielded data which are in close agreement with those derived from similar studies using the structurally dissimilar mixed agonist-antagonist [^{3}H]dihydroergocryptine as a binding probe (9, 10). The number of binding sites per platelet derived from these studies (100–220) is very close to that noted in the present study (260 ± 95). In addition, the dissociation constants and order of potency for a wide variety of ligands obtained from experiments measuring the inhibition of binding induced by these ligands are similar. These observations suggest that [^{3}H]phentolamine and [^{3}H]dihydroergocryptine interact with a common platelet membrane site which is also capable of binding α-adrenergic agonists and antagonists and fulfills the pharmacological criteria of the α-adrenergic receptor, although the mechanism by which ligands of such dissimilar structure can interact with this common binding site remains unclear.

As noted by Alexander *et al.* (10), [^{3}H]dihydroergocryptine may have limitations as a probe for α-receptor binding studies because it is slowly bound and only incompletely dissociable. In this regard, the rapid and completely reversible binding noted with [^{3}H]phentolamine may be an asset for α-receptor studies, because physiological hormone-receptor interactions are known to be rapid events. On the other hand, studies using [^{3}H]phentolamine as the binding probe are complicated by the fact that a large fraction of [^{3}H]phentolamine binding appears to be nonspecific, *i.e.*, not displaced by epinephrine and therefore presumably not on α-receptors. Thus, small changes in binding may be obscured by the large background of nonspecifically bound ligand. The magnitude of this problem is even greater when studies of binding to intact cells are attempted because the concentration of specific binding sites (i.e. "receptors") in these assays may be limited and [^{3}H]phentolamine may be taken up by the cells. It is not surprising, therefore, that we have so far been unsuccessful in using [^{3}H]phentolamine to characterize the α-receptor on intact platelets.

Recently, U'Prichard and Snyder (29) reported that GTP inhibited the binding of α-adrenergic agonists but not antagonists in calf brain membranes. We have also noted that GTP decreased the ability of epinephrine to inhibit [^{3}H]phentolamine binding to human platelet membranes, indicating that the affinity of epinephrine binding to the α-receptor had been lowered by the presence of GTP. No change in [^{3}H]phentolamine binding was observed. The finding that epinephrine affinity was *decreased* by GTP would seem paradoxical because epinephrine-induced inhibition of platelet adenylate cyclase (an α-adrenergic event) requires the presence of GTP (30, Steer & Wood, submitted for publication). It is tempting to speculate that this apparent paradox indicates that the α-receptor-adenylate cyclase complex possesses separate regulatory sites for GTP that modulate receptor binding and which allow for adenylate cyclase inhibition. Distinct guanine nucleotide sites have been reported to regulate glucagon binding to liver membranes and adenylate cyclase stimulation by the hormone (31). Thus, the presence of separate guanine nucleotide sites regulating receptor and cyclase behavior may be a widespread phenomenon.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Dr. W. Rein of Ciba-Geigy for help in obtaining [^{3}H]phentolamine.

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